A. Formalities

The Examiner states that the inventors' declaration is defective because the address for inventor Donald P. Weeks was changed without initialing and dating the change. Being submitted herewith is a new inventors' declaration under 37 C.F.R. § 1.67 which Dr. Weeks has signed and dated.

B. Written Description Rejection

The Examiner has rejected Claims 1, 4, 7, 21, 24, 36, 39, 44, 47 and 48 under section 112 as containing subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the art that the inventors had possession of the claimed invention at the time the application was filed. Applicants respectfully traverse this rejection.

As noted by the Examiner, Applicants teach a specific nucleic acid sequence (SEQ ID NO:3) isolated from *Pseudomonas maltophilia*, strain DI-6, encoding a dicamba-degrading oxygenase (SEQ ID NO:4). Applicants also provide substantial additional disclosure in the application describing how to identify and isolate additional nucleic acid sequences encoding the same or different oxygenases. See, *e.g.* page 9, lines 18-28, page 10, line 10 through page 11, line 4, and Examples 1 and 2. As described in more detail below and in paragraph 2 of the accompanying Weeks Declaration, Applicants have identified several additional genes encoding dicamba-degrading oxygenases using the procedures described in the application. In particular, the new genes were identified using a fragment of the *P. maltophilia* DI-6 oxygenase gene as a probe, as taught in the application. The newly-identified genes are greater than 90% identical to SEQ ID NO:3.

Applicants have further provided extensive disclosure in the present application of how to make and use transgenic hosts, including bacteria and plants. See, *e.g.* page 11, line 30 through page 25, line 6 of the application. As described in more detail in paragraphs 3 and 4 of the Weeks Declaration, Applicants have prepared transgenic hosts (bacteria and plants) producing active dicamba-degrading oxygenase. This was accomplished utilizing the teachings of the present application.

For the foregoing reasons, it is submitted that the specification of the present application clearly conveys that Applicants were in possession of the claimed subject matter at the time the application was filed. Accordingly, the Examiner is asked to withdraw this rejection

C. Enablement Rejection

The Examiner has rejected Claims 39, 44, 47 and 48 under section 112 for lack of enablement. It is the Examiner's position that: "The state of the art for modification of gene expression or of phenotypic characteristics in plants by genetic transformation is highly unpredictable and hence significant guidance is required to practice the art without undue experimentation." Applicants respectfully traverse this rejection.

Contrary to the Examiner's contention, Applicants have provided extensive guidance as to how to produce transgenic hosts, including plants, comprising a DNA sequence encoding a dicamba-degrading oxygenase. See, *e.g.* page 11, line 30 through page 25, line 6 of the application. This guidance includes promoters, other expression control sequences, vectors, constructs, methods of transformation, methods of selection, etc.

Further, ultilizing the teachings of the application, Applicants have produced transgenic plants that express the *P. maltophilia* DI-6 oxygenase (SEQ ID NOS: 3 and 4). See paragraph 4 of the Weeks Declaration. In particular, Applicants have produced transgenic tobacco and *Arabidopsis* plants comprising DNA encoding the *P. maltophilia* DI-6 oxygenase. See paragraph 4 of the Weeks Declaration. The transgenic plants have been shown to express active oxygenase protein.

For all of the foregoing reasons, the Examiner is asked to withdraw this rejection.

D. Enablement Rejection

The Examiner has rejected Claims 1, 4, 7, 21, 24, 36, 39, 44, 47 and 48 under section 112 for lack of enablement. It is the Examiner's position that: "the specification, while being enabling for the DNA molecule of SEQ ID NO: 3 encoding the dicamba-degrading oxygenase of SEQ ID NO:4, does not reasonably provide enablement for any DNA molecule encoding a dicamba-degrading oxygenase." Applicants respectfully traverse this rejection.

Contrary to the Examiner's contentions, Applicants have provided substantial guidance as to how to obtain additional DNA molecules encoding dicamba-degrading oxygenases in addition to the one specific molecule whose sequence (SEQ ID NO:3) is given in the application. See, *e.g.* page 9, lines 18-28, page 10, line 10 through page 11, line 4, and Examples 1 and 2 of the present application. This includes guidance as to the selection and preparation of probes and the sequence of one DNA molecule (SEQ ID NO:3) encoding a dicamba-degrading oxygenase (the *P. maltophilia* strain DI-6 oxygenase). Also, contrary to the Examiner's contentions, techniques for identifying and isolating DNA molecules, including the design of suitable probes and PCR conditions, are well known in the art. See, *e.g.*, Maniatis et al., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor, NY (1982); Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor, NY (1989); and *PCR Protocols: A Guide To Methods And Applications* (Innis et al. editors, Academic Press, Inc., 1990).

The Examiner states: "Applicant admits at page 54, lines 3-6, that Applicant's SEQ ID NO:3 only showed a 33.8% homology to another oxygenase component of a vanillate demethylase." The discussion on page 53, line 30 through page 54, line 6, of the application only shows that the dicamba-degrading oxygenase (SEQ ID NO:4) of the present invention was not known prior to the present invention and was not closely related to known oxygenases. It does not mean that additional dicamba-degrading oxygenases do not exist or will be difficult to identify and isolate. Indeed, it is submitted that, with the guidance provided by Applicants, doing so will now be a matter of routine experimentation.

In fact, using the procedures described in the application, Applicants have identified several additional dicamba-degrading oxygenases. See Weeks Declaration, paragraph 2. In particular, new dicamba-degrading bacterial strains were isolated, and Applicants used a portion of the DNA encoding the *P. maltophilia* DI-6 oxygenase (SEQ ID NO:3) as a probe to identify the new oxygenases in these new bacterial strains, all as taught in the application. The new dicamba-degrading oxygenases are greater than 90% identical to the *P. maltophilia* DI-6 oxygenase.

For all of the foregoing reasons, this rejection should be withdrawn.

E. Indefiniteness Rejections

The Examiner has rejected Claims 1-7, 21-24, 36-39, 44, 47 and 48 as being indefinite for a variety of reasons. It is submitted that most of the bases of this rejection have been overcome by the above amendments.

Applicants have not amended Claim 7 as indicated by the Examiner because it is submitted that the meaning of "vector" would be clear to those skilled in the art. Also see page 16, lines 17-28 of the present application.

Applicants also have not amended Claim 39 as indicated by the Examiner. The term "tolerant" is defined on page 18, line 25 through page 19, line 2 of the present application. Therefore, its meaning in Claim 39 is clear.

In view of the foregoing, the Examiner is requested to withdraw this rejection.

CONCLUSIONS

It is submitted that the pending claims are in condition for allowance, and a speedy allowance of them is requested.

Respectfully submitted,

SHERIDAN ROSS P.C.

By: Wannell Crook

Wannell M. Crook
Registration No. 31,071
1560 Broadway, Suite 1200
Denver, CO 80202-5141
(303) 863-9700

Date: July 27, 2001

VERSION OF CLAIMS SHOWING CHANGES

Claims 8-20, 25-35, 40-43, 45-46 and 49 have been canceled.

Claims 1-2, 4-5, 21-22, 36-37, 44 and 47-48 have been amended as follows:

1. (Amended Once) An isolated DNA molecule comprising a DNA sequence [coding for] encoding a dicamba-degrading oxygenase.

2. (Amended Once) The DNA molecule of Claim 1 comprising a DNA sequence [coding for] encoding a dicamba-degrading oxygenase having the amino acid sequence of SEQ ID NO:4.

4. (Amended Once) A DNA construct comprising a DNA sequence [coding for] encoding a dicamba-degrading oxygenase operatively linked to expression control sequences.

5. (Amended Once) The DNA construct of Claim 4 comprising a DNA sequence [coding for] encoding a dicamba-degrading oxygenase having the amino acid sequence of SEQ ID NO:4.

21. (Amended Once) A transgenic host cell comprising DNA [coding for] encoding a dicamba-degrading oxygenase, said DNA being operatively linked to expression control sequences.

22. (Amended Once) The transgenic host cell of Claim 21 wherein the DNA [codes for] encodes a dicamba-degrading oxygenase having the amino acid sequence of SEQ ID NO:4.

36. (Amended Once) A transgenic plant or part of a plant comprising one or more cells comprising DNA [coding for] encoding a dicamba-degrading oxygenase, said DNA being operatively linked to expression control sequences.

37. (Amended Once) The transgenic plant or plant part of Claim 36 wherein the DNA [codes for] encodes a dicamba-degrading oxygenase having the amino acid sequence of SEQ ID NO:4.

44. (Amended Once) A method of controlling weeds in a field containing a transgenic plant according to any one of Claims [36-43] 36-39 comprising applying an amount of dicamba to the field effective to control the weeds in the field.

47. (Amended Once) A method of selecting transformed plant cells comprising:

providing a population of plant cells;

transforming at least some of the plant cells in the population of plant cells with a DNA construct according to any one of Claims [4-16] 4-7; and

[growing] selecting the transformed plant cells by culturing the resulting population of plant cells in a culture medium containing dicamba at a concentration selected so that transformed plant cells [will grow] proliferate and untransformed plant cells [will not grow] do not proliferate.

48. (Amended Once) A method of selecting transformed plants comprising:

providing a population of plants which may comprise one or more plants comprising [suspected of comprising] a DNA construct according to any one of Claims [4-16] 4-7; and

selecting transformed plants by applying an amount of dicamba to the population of plants selected so that transformed plants [will] grow, and growth of untransformed plants [will be] is inhibited.

Tobacco Transformation Protocol

Preparation of Tobacco explants:

Select 3-4 inch leaves from 1 month old plants (top 2 leaves). This is sufficient for 30-40 explants. Place the leaves into a large beaker and cover with dH_20 for 20-30 minutes. Drain the water and cover with 10% Chlorox™ plus Tween 20™ (1 drop per 50 ml). Allow to soak for 15 minutes. Rinse 3X-5X with sterile dH_2O.

Trim off the out side margin of the leaf along with the tip and petiole. Dissect out the midrib and slice the remaining tissue into 0.5 X 0.5 cm squares.

Preculture the explants, 30 per plate, adaxial side up, for 1 day prior to inoculation.

Preculture medium (PM)

MS salts with B5 vitamins, 3% sucrose amended with 1 mg/l BAP, 0.1 mg/l NAA and 8 µg/l pCPA (p-Chlorophenoxyacetic acid), pH 5.7 (Note: filter sterilize all growth regulators)

Agrobacterium inoculum preparation:

Initiate a 2 ml culture of Agrobacterium in YEP medium (10 g/l peptone, 10 g/l NaCl and 5 g/l yeast extract, pH 7.0) amended with appropriate antibiotics. Allow the culture to become saturated. Subculture the 2 ml culture into 50 mls of YEP medium and allow to grow for 6-8 hrs (28 °C with constant shaking). Harvest the cells by centrifugation (3,000-4,000 rpm). Resuspend the bacterial pellets to a final OD 660 = 0.3-1.0, in co-cultivation medium.

Co-cultivation medium (CM):

1/10 MS/B5 medium, amended with 3% sucrose, 1.0 mg/l BAP, 0.1 mg/l NAA and 8 µg/l pCPA.

Transfer the prepared Agrobacterium inoculum to petri plates. Inoculate the pre-cultured explants for 30 minutes. Blot the explants on sterile filter paper and place on to co-cultivation medium (10 explants per plate) solidified with 0.8% agar, which is over laid with a sterile filter paper(Co-culture explants adaxial side up. Allow the explants to co-cultivate for 3 days.

Following the co-cultivation period briefly wash the explants in regeneration medium (RM).

Regeneration medium (RM)

MS/B5 medium amended with 1.0 mg/l BAP, 0.1 mg/l NAA and 3% sucrose, pH 5.7. Utilize the antibiotics carbenicillin (500 mg/l) and cefotaxime (100 mg/l).

Blot the washed explants on sterile filter paper. Transfer the tissue (10 explants per plate) to RM medium solidified with 0.8% agar, plus the appropriate selective agent (Kanamycin 150 mg/l). Culture the explants adaxial side up. Transfer the tissue every 2 weeks to fresh RM medium.

Excise shoots (>3 cm) and subculture to rooting medium.

Rooting medium
MS salts with full strength B5 vitamins, amended with 0.1 mg/l NAA, and 1% sucrose. Solidify the medium with 0.8% agar. Maintain the antibiotics (if using npt II 75 mg/l) carbenicillin (500 mg/l) and cefotaxime (100 mg/l).

Acclimate rooted shoots to soil.

SOUTHERN BLOT

Reagents

0.25 N HCl
900 ml H_2O
20.7 ml HCl (12.1 N)
Bring to 1 L, autoclave (fill bottle half full).

0.5 N NaOH/1.5M NaCl
300 ml 5M NaCl stock
20 g solid NaOH
Bring to 1 L

0.5M Tris-HCl/1.5M NaCl
500 ml 1 M Tris HCl stock (pH 7.0)
300 ml 5M NaCl stock
100 ml H_2O
Bring to 1L.

20x SSC stock
175.32 g NaCl
88.23 g Sodium Citrate
800 ml H_2O
pH to 7.0 with HCl. Bring to 1L. autoclave (fill bottle half full).

5x Blocking Buffer (500 ml)
400 ml H_2O (in 1 L flask)
5.8 g Maleic Acid (.1M)
4.375 g NaCl (150 mM)
3.75 g NaOH
Bring to pH 7.5 before adding blocking agent.
Add 50 g Blocking agent
Heat gently (do not exceed 60 C)with stirring so that blocking reagent goes into solution. Adjust pH again to 7.5, but only with NaOH. **Do not add acid** (the blocking reagent will precipitate out of solution). Adjust volume to 500 ml. **Autoclave only 15 minutes.** Cool and dispense 40 ml/50 ml Falcon tube. Freeze at -20 C.

Prehybridization/Hybridization Solution (50 ml)
12.5 ml 20x SSC stock
100 ul 10% SDS (Lauryl Sulfate) stock
500 ul 10% N-Lauroylsarcosine stock
5 ml 5x Blocking Buffer
31.9 ml water

2xSSC/0.1% SDS
100 ml 20 x SSC stock
10 ml 10% SDS stock
800 ml H_2O (filtered)
Bring to 1 Liter

0.1xSSC/0.1% SDS
5 ml 20 x SSC stock
10 ml 10% SDS stock
900 ml H_2O (filtered)
Bring to 1 Liter.

Washing Buffer
900 ml 1x Maleate Buffer (approx.)
2.7 ml Tween 20 (a 0.3% conc.)

Blocking Buffer 2

Dilute 5x Blocking Buffer with 1x Maleate buffer to make 1x Blocking Buffer.
For 50 ml:
10 ml 5x Blocking Buffer
40 ml 1x Maleate Buffer

Genius Buffer 3 (10x)

Note: Dilute to 1x with filtered water before using.
121.1 g Tris (0.1M)
58.44 g NaCl (0.1 M)
pH to 9.5 with NaOH. Bring to volume and autoclave. Fill bottle half full.

10x Maleate Buffer (1L)

Note: Dilute to 1x with filtered water before using.
116.1 g maleic acid
87.7 g NaCl
800 ml H_2O
Dissolve, then bring to volume. pH to 7.5 with solid NaOH (about 160 g for 2L).
Autoclave -- fill bottle half full.

Gel

(Medium size, 14 lanes, thin comb)
Use 100 ml of 1.0% agarose for gel.
Marker lanes - The amount of marker used depends on exposure time. If exposing for 1 hour, 3 ul of DIG III marker should be added to 17 ul of H_2O and 5 ul of SALB dye. If exposing for 15 minutes, add 9 ul of DIGIII marker to 11 ul of H_2O and 5 ul of SALB dye.

Transfer

1. Cut lower left corner of the gel with a razor blade to distinguish between the front and back. Flip gel before starting depurination step.

2. Depurinate by a 10 minute wash in 250 ml of 0.25 N HCl on rotary shaker at room temperature (Time should not exceed ten minutes). Drain HCl solution from dish.

3. Add water to dish and rinse gel briefly. Drain water from dish and add fresh water. Rinse again briefly, drain water, and add denaturing solution.

4. Denature by two 15 minute washes in 250 ml of 0.5N NaOH/1.5M NaCl on rotary shaker at room temperature. Drain denaturing solution from dish and add neutralizing solution.

5. Neutralize by two 15 minute washes in 250 ml of 0.5 M Tris-HCl (pH 7.0)/1.5M NaCl on rotary shaker at room temperature.

6. Cut a stack of paper towels(approx. 7 cm high) that are 10 x 13 cm in size. Also cut from Whatman paper the following sizes:

1	15 x 42 (wick)
3	13 x 15
3	10 x 13

Cut 1 membrane 11 x 14 cm.

7. Wet membrane by placing in dish containing filtered water, then transfer to dish containing 20x SSC. Wet the wick and the pieces of Whatman paper (together) in a dish containing a small amount of 20x SSC immediately before use.

8. Set up transfer apparatus in glass dish:

From bottom: gel tray, wick, three layers of Whatman, gel (inverted), membrane, three more layers of Whatman. Cover the apparatus with a layer of plastic wrap, cutting a window smaller than the size of the top layer of Whatman with a razor blade. Note: when cutting window make sure the plastic wrap has no tears in it. Paper towels, glass plate and light weight (200 g to 400 g).

Note: There should be no bubbles between layers. These can be eliminated by rolling a 10 ml pipette back and forth over the surface.

Note: Cut the membrane at the lower right corner so that the cut corners of the gel and membrane coincide during the transfer process.

Note: Be careful to orient the membrane on the gel correctly. Do not pick the membrane up off the gel and lay it down again. Detectable transfer can take place immediately. If you make a mistake, cut another piece of membrane and try again.

9. Transfer in 20xSSC overnight. Dish should be one-third full of 20xSSC.

10. Remove blot from transfer apparatus with forceps. Place on fresh piece of Whatman with DNA side up. U/V cross-link membrane using auto setting on Stratalinker. Start prehybridization immediately or cover the blot with another piece of Whatman paper and wrap in aluminum foil. Store dry at room temperature in a dessicator. If blot has been dried, wet it a few minutes in prehybridization solution in a dish to saturate it before placing it in the hybridization bag.

Hybridization

1. Pipette prehybridization solution into a plastic bag (20 ml solution per 100 cm^2 of membrane surface area). Place blot in bag, DNA side up, and seal without bubbles. Prehybridize membrane for a minimum of two hours at 68 C in Hot Shaker.

2. Denature probe by placing it in boiling water bath for 10 minutes or 68 C for 10 minutes if the solution contains formamide. After boiling immediately place probe on ice.

3. If probe is not already dissolved in hybridization solution, prepare it the same as the prehybridization solution. (For a 100 cm^2 blot (10 x 10 cm), use a minimum of 10 ml of fresh hybrization solution. For a 400 cm^2 blot (20 x 20 cm), use a minimum of 25 ml of fresh hybridization solution.) Add the probe to the hybridization solution so that the concentration is 5 ng/ml.

4. Drain prehybridization solution from bag. Add the hybridization solution to a new bag containing blot, seal without bubbles, and hybridize overnight at 68 C in the Hot Shaker.

5. Drain hybrization solution from bag into capped Falcon sterile tube. **REMEMBER TO KEEP PROBE.** This solution may be stored for one year at -20 C and reused(To reuse, thaw and denature by heating by boiling for 10 minutes). Remove membrane from

bag and wash briefly, DNA side up, in 2x SSC/0.1% SDS in dish. Drain and add fresh solution.

6. Wash membrane twice, DNA side up, in 2xSSC/0.1% SDS at 65 C for 10 minutes each washing in Hot Shaker. Drain solution and add 0.1xSSC/0.1% SDS solution.

7. Wash membrane twice, DNA side up, in 0.1 SSC/0.1% SDS at 65 C for 20 minutes each washing in Hot Shaker.

Blocking Membrane

1. Equilibrate membrane in washing buffer in a dish freshly rinsed with water for 1 minute at room temperature on rotary shaker.

2. Transfer membrane to bag and pipette 30 ml of Blocking Buffer 2 into bag. **For sufficient blocking, there must be ample room to allow for unrestricted shaking of the membrane.** Seal bag without bubbles. Incubate for a minimum of 1 hour at room temperature on rotary shaker. Save 30 ml of Blocking Buffer 2 for next step.

3. Prepare antibody solution. Spin the antibody for 30 seconds in microfuge to pellet particulate matter. Dilute 3 ul of anti-DIG alkaline phosphatase into 30 ml of Blocking Buffer 2 (1:10000 dilution).

4. Drain bag and discard solution. Replace with antibody solution. Seal bag without bubbles.

5. Incubate for 30 minutes at room temperature on rotary shaker.

6. Discard antibody solution and remove membrane from bag. Place membrane in freshly rinsed dish containing washing buffer and wash twice at room temperature for 15 minutes on rotary shaker. Drain solution and add 1x Genius Buffer 3.

7. Equilibrate membrane in 1x Genius Buffer 3 for 2 minutes at room temperature on rotary shaker.

Chemiluminescent Substrate

1. Place the membrane, DNA side up, between two sheets of acetate (plastic transparency sheets). Gently lift the top sheet of plastic and, with a sterile pipette, add approximately 1 ml of CSPD to the top surface (DNA side) of the membrane, scattering drops of CSPD over the surface. Rock the membrane gently to distribute the reagent over the surface. Lower the top sheet of plastic and spray lightly with alcohol. Then, with a damp Kimwipe, gently wipe the top sheet, being sure to remove all bubbles present on the sheet and to create a liquid seal over the membrane. Tape the sides of the plastic sheets so that the membrane doesn't dry out, especially if you are going to expose it again the next day.

2. In the darkroom, expose the covered membrane to X-ray film at room temperature (no intensifying screens). Light intensity increases for 7-8 hours, is maximal for 12 hours, and then slowly decreases. The recommended initial exposure time for single copy gene detection in a genomic Southern blot hybridization (10 ug in human genomic DNA) is 60 minutes at room temperature. The same gene can be detected in as little as 15 minutes if the X-ray film exposure is performed 8-24 hours after application of CSPD. Shorter and longer exposure times can be used to obtain optimal signal intensity.

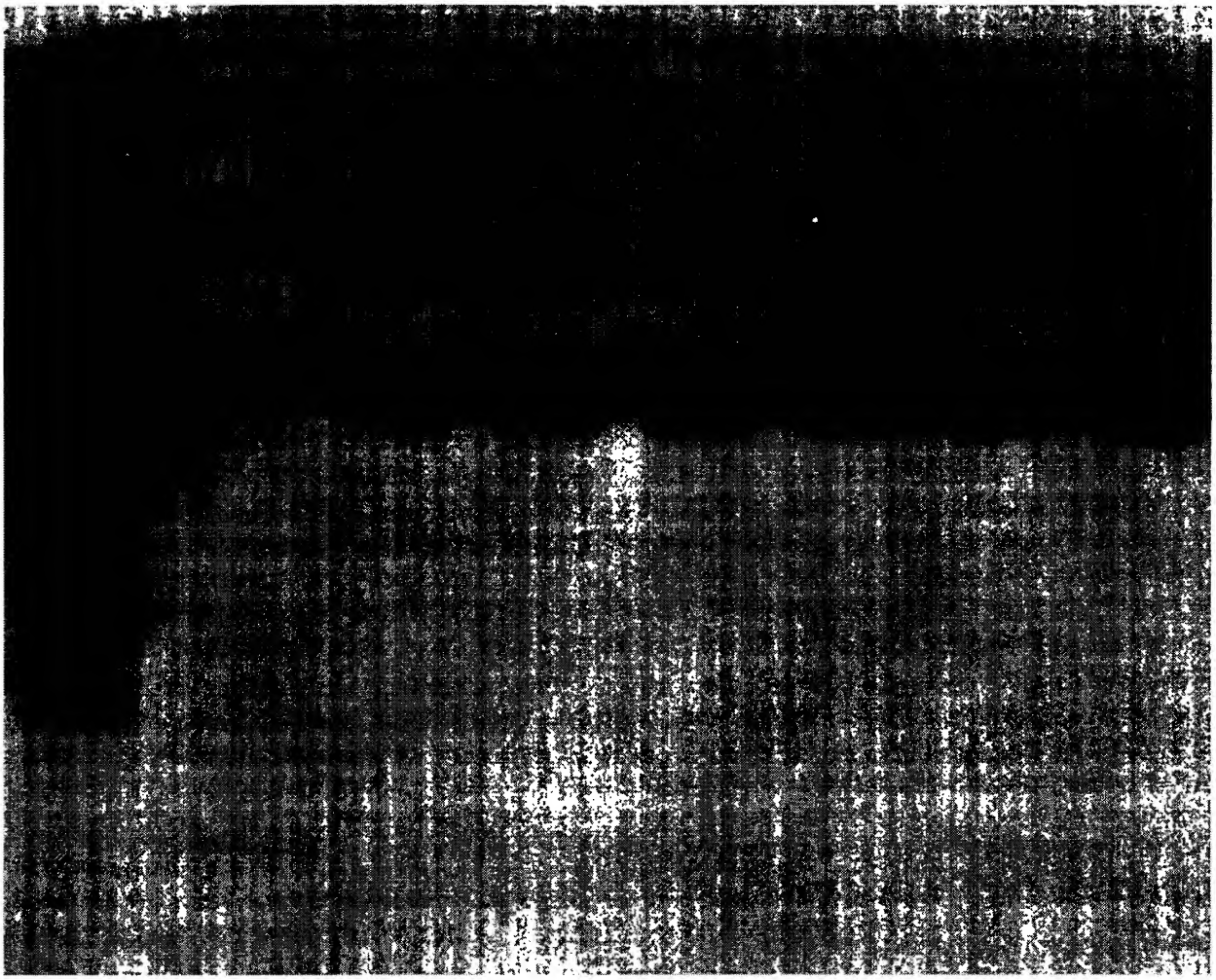
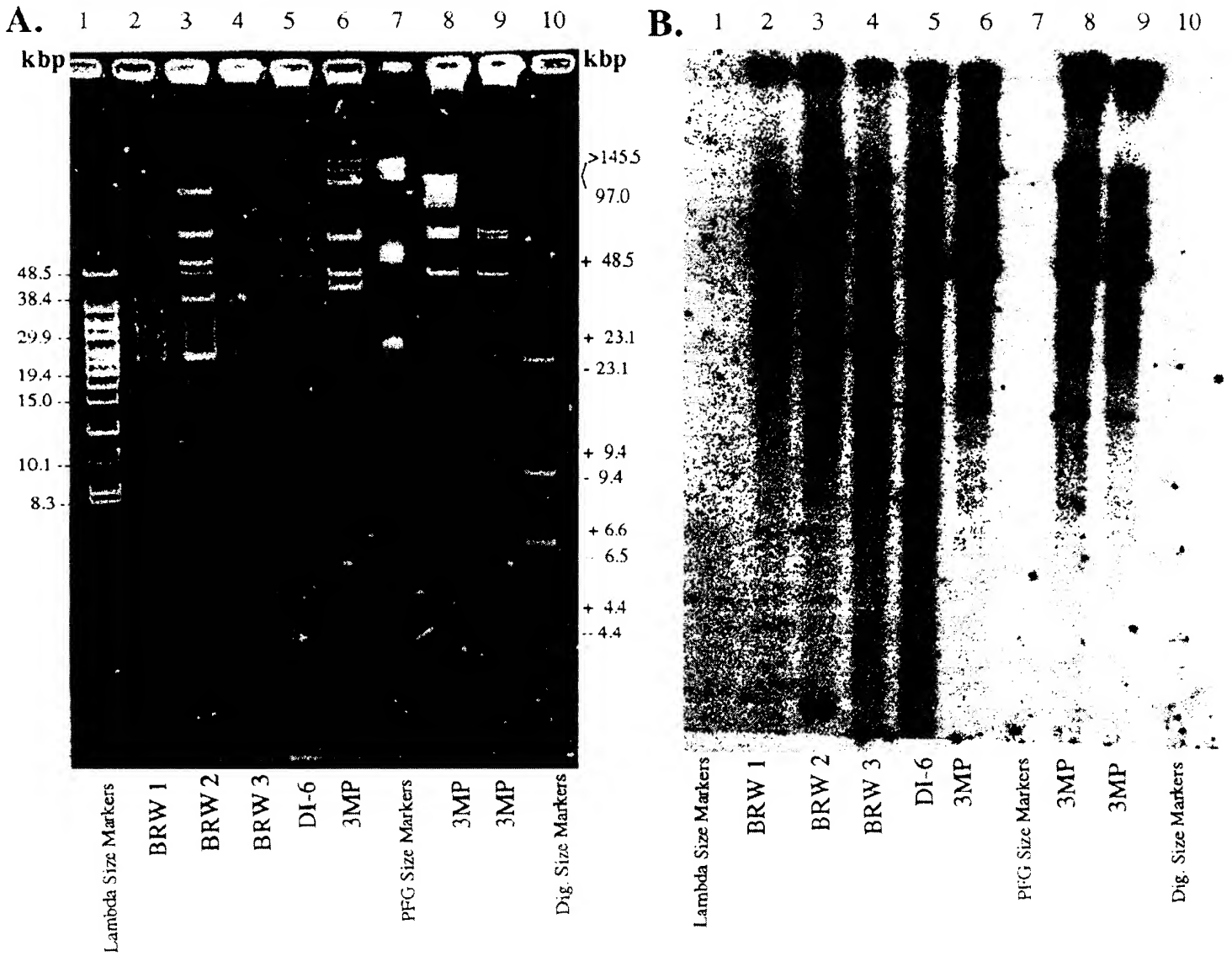


Figure C

Comparison of Plasmids from Dicamba-degrading Bacteria Detected with Ethidium Bromide (A) and Chemiluminescence (B) after Being Probed with Oxygenase Gene Probe (1 Hour Exposure)



IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In Re the Application of:	Group Art Unit: 1638
WEEKS et al.	Examiner: D. Kruse
Serial No.: 09/055,145	
Filed: April 3, 1998	DECLARATION OF DR. DONALD P. WEEKS
Atty. File No.: 3553-18	
For: "METHODS AND MATERIALS FOR MAKING AND USING TRANSGENIC DICAMBA-DEGRADING ORGANISMS"	

I, Dr. Donald P. Weeks, declare that:

1. I am the same Donald P. Weeks who is named as an inventor on the above-referenced patent application.

2. Experiments have been performed in my laboratory to identify additional genes encoding dicamba-degrading oxygenases. In particular, we have isolated additional dicamba-degrading bacteria and identified several copies of genes encoding dicamba-degrading oxygenases in each of them. These experiments were performed as follows.

a. Five bacterial strains were used in these studies:

(1) *Pseudomonas maltophilia* strain DI-6 was obtained from Douglas Cork, Illinois Institute of Technology, Chicago, Illinois.

(2) Three of the strains were isolated by our laboratory from soil taken from various locations in a storm water retention pond at the dicamba manufacturing plant in Beaumont, Texas (*Pseudomonas* sp. BRW 1, *Sphingomonas* sp. BRW 2, *Pseudomonas* sp. BRW 3). These strains were isolated by plating serial dilutions (in sterile water) of the soil samples on reduced chloride medium ("RCI"; Krueger et al., *J. Agric. Food Chem.*, **37**, 534-538 (1989)) containing 5 mM dicamba as the sole carbon source and incubating the plates at 30°C. Several dozen strains capable of utilizing dicamba as a sole carbon source were isolated, but some isolates, which

formed large colonies on agar media, did not grow in liquid culture, and were not retained for further studies. The three strains used for these studies (*Pseudomonas* sp. BRW 1, *Sphingomonas* sp. BRW 2, *Pseudomonas* sp. BRW 3) grow rapidly in liquid culture.

(3) Strain 3MP is *Escherichia coli* strain DH5α transformed with DNA encoding the *P. maltophilia* strain DI-6 dicamba-degrading oxygenase (for preparation, see paragraph 3.a. below).

(4) All five of these strains of bacteria are dicamba-degrading bacteria that can utilize dicamba as the sole carbon source.

b. The bacteria were grown on plates containing gelrite (10 g/L) and RCl medium with 5 mM dicamba. Single colonies were inoculated in 5 milliliters (ml) liquid RCl medium containing 5 mM dicamba (duplicate cultures were grown). The cultures were grown in 15 ml sterile test tubes with shaking at 225 RPM at 30°C to saturation density, which required between 48-72 hours. Two ml of each culture were inoculated into 25 ml RCl containing 5 mM dicamba in a 125 ml flask. The cultures were grown to saturation density, which again required approximately 48-72 hours. At this point a pink by-product became visible in the medium. Ten ml of each of these cultures were inoculated in 500 ml RCl containing 5 mM dicamba in one-liter flasks. The cultures were grown at 30°C with shaking at 225 RPM to saturation density, approximately 48 hours. The cultures were centrifuged at 4°C in a Beckman J-25I centrifuge at 5000XG for 10 minutes while maintaining a sterile environment. The supernatant was poured off and the pellets were resuspended in 10 ml RCl. Once resuspended, the 10 ml were placed into a one liter flask with 500 ml RCl containing 5 mM dicamba and incubated for approximately 48-72 hours as described above. The cells were then centrifuged in a Beckman J-25I centrifuge at 4°C at 5000XG for 15 minutes.

c. A Qiagen® plasmid purification kit (Qiagen, Inc.) was used to isolate the plasmids using the Very Low-Copy Plasmid protocol (Qiagen® tip-100). The plasmid DNA was precipitated by dividing the plasmid DNA from each strain into two tubes. One tenth volume of 3M sodium acetate and two volumes of 100% ethanol (EtOH) were added to each tube. The tubes were incubated overnight at -20°C. They were then centrifuged in a microfuge at 4°C for 15 minutes. The supernatant was poured off. Five hundred µl of 70% cold EtOH were added to each tube. The

tubes were centrifuged in the microfuge at 4° C for 15 minutes at 4,000 X G. The supernatant was poured off, and the pellet was dried for one hour. DNA was resuspended in 50 µl TE buffer (0.01 M Tris, 0.001 M EDTA), pH 8.0.

d. In order to prepare the samples for CHEF (contour-clamped homogenous electric field) gel electrophoresis analysis, TE buffer, pH 8.0, was added to the samples with 1x SALB dye (1.5 mg/ml bromophenol blue, 0.5x TBE (Tris-borate-EDTA buffer, 90 mM Tris-borate, 2 mM EDTA), 0.6 g/ml sucrose, 4 µl of 10mg/ml boiled RNase/ml). The molecular size markers used were the high molecular weight markers (Gibco BRL) (15 µl marker, 5 µl TE, pH 8.0, 5 µl SALB dye) (which was placed at 55°C for 10 minutes immediately before use), the Low Range PFG markers (Biolabs) (0.25 µg marker sealed with molten agarose), and the DIG Marker II (Boehringer Mannheim GmbH) (15 µl marker, 5 µl H_2O). The prepared samples were loaded into wells of a 0.7% agarose gel in a BioRad CHEF gel apparatus. Controls on the CHEF gel apparatus were set as follows: initial switch time (2 seconds), final switch time (2 seconds), run time (10 hours), and voltage (6V). Two liters of 1x TAE (0.04 M Tris-acetate, 0.001 M EDTA) was added to the CHEF gel apparatus. Following the run time, the gel was soaked for approximately 30 minutes in 1µg/ml ethidium bromide solution. The stained gel was photographed under UV light using a Fotodyne Gel Documentation apparatus.

e. Digoxygenin-labeled probes were prepared using PCR amplification of a section of the oxygenase gene (Genius System User's Guide For Membrane Hybridization). The resulting probe was approximately 900 bp in length. The PCR products were checked by loading the samples onto a 1% agarose gel. PCR primers for the oxygenase gene were:

forward - 5'-GCTGCCCGAGGAACTGTCCGAAAAG-3'; and

reverse - 5'–CGACGACGACCTTGTCCTCCTTGAC -3'.

f. Plasmid DNA was transferred to a nylon membrane using the Southern blot technique. The Southern blot of plasmid DNA was probed with the digoxygenin-labeled oxygenase probe. Stringent hybridization and membrane wash conditions were used. Chemiluminescence (Roche, Genius Nouradioactive Detection Kit) was used to detect hybridization. Five-minute, 1-hour, and 24-hour exposures were performed. A complete description of the protocol and reagents

for preparing the Southern blots is attached hereto as Exhibit 1, the entire contents of which are incorporated herein by reference.

g. The results are shown in attached Figures A and B:

(1) Figure A shows plasmid DNAs from the five bacterial strains [*Pseudomonas maltophilia*, strain DI-6 (DI-6), *Pseudomonas* species, strain BRW 1 (BRW 1), *Sphingomonas* species, strain BRW 2 (BRW 2), *Pseudomonas* species, strain BRW 3 (BRW 3), and *Escherichia coli*, strain 3MP (3MP)] stained with ethidium bromide. Migration positions of various molecular weight size markers are designated on the left and right sides of the photographed gel image.

(2) Figure B shows hybridization of plasmid DNAs on a Southern blot of the CHEF gel pictured in Figure A with the digoxygenin-labeled PCR-amplified fragment of the cloned strain DI-6 oxygenase gene. The photographed X-ray film image shows chemiluminescence emanating from the hybridized oxygenase gene probe. The exposure time was 1 hour. As can be seen, the oxygenase probe hybridized strongly to multiple bands from each bacterial strain, indicating close similarities between the probe and several bacterial genes (greater than 90% identity given the stringent hybridization conditions and the size of the probe) and between the various bacterial genes. From these results, it was concluded that each of the three newly-discovered strains (BRW 1, BRW 2 and BRW 3) contains at least one dicamba-degrading oxygenase gene. The reason for hybridization of the probe to multiple bands from each strain is that the bacteria contain several "megaplasmids," and several of these contain at least one copy of an oxygenase gene.

3. Transgenic *E. coli* strain 3MP expressing the *P. maltophilia* strain DI-6 oxygenase has been produced in our laboratory.

a. As noted in paragraph 2, transgenic *E. coli* strain 3MP was prepared by transforming *E. coli* strain DH5α with DNA encoding a dicamba-degrading oxygenase. More specifically, *E. coli* strain DH5α was transformed with plasmid DNA isolated from *P. maltophilia* strain DI-6 by the methods described in paragraph 2.c. above. *E. coli* strain DH5α cultures were

made competent for transformation by the methods described in Sambrook et al., *Molecular Cloning*, pages 1.74-1.84 (2nd edition, Cold Spring Harbor Press, 1989).

b. As also noted in paragraph 2, *E. coli* strain 3MP can utilize dicamba as its sole carbon source. *E. coli* DH5α, the parent strain of *E. coli* 3MP, cannot grow on dicamba under the same conditions as the transgenic 3MP strain. Thus, it is the presence of the *P. maltophilia* strain DI-6 oxygenase and associated dicamba-degrading genes from *P. maltophilia* DI-6 in it that allows *E. coli* strain 3MP to degrade dicamba and utilize it as a carbon source.

c. As shown in Figure B, the *P. maltophilia* strain DI-6 oxygenase probe hybridized with plasmid DNA from *E. coli* strain 3MP, indicating the presence of DNA encoding the DI-6 oxygenase in strain 3MP.

d. The oxygenase produced by transgenic *E. coli* strain 3MP was purified and mixed with purified ferredoxin and reductase components (purified from *P. maltophilia* DI-6 as described in Example 1 of the above-referenced application). This combination produced dicamba O-demethylase activity. The combination of just the ferredoxin and reductase did not produce dicamba O-demethylase activity. This assay was performed as described in Example 1 of the above-referenced application.

4. Transgenic tobacco plants expressing the *P. maltophilia* strain DI-6 oxygenase have also been produced.

a. First, the gene encoding the oxygenase component of dicamba O-demethylase of *P. maltophilia* DI-6 (isolation described in Example 2 of the above-referenced application) was placed into a cassette suitable for expression in plants as follows. Oligonucleotide primers were designed to generate a Nco I site at the 5' end and an Xba I site at the 3' end of each strand of the oxygenase gene by PCR amplification. The authenticity of the resulting PCR product was confirmed by sequencing the PCR amplified gene. This gene was then cloned into the Nco I and Xba I sites of the polylinker region of the pRTL2 vector. The pRTL2 vector was provided by Dr. Tom Clemente of the University of Nebraska Plant Transformation Core Research Facility, Lincoln, Nebraska. This vector contains a 144 bp translation enhancer sequence from tobacco etch virus (TEV) (Carrington and Freed, *J. Virology*, **64**:1590-1597 (1990)) at the 5' end of the polylinker. The oxygenase gene with the 5' TEV translation enhancer was then cloned as a Xho I/Xba I fragment into

the plant expression vector pKLP36 obtained from Indu Maiti, University of Kentucky. This binary vector contains the peanut chlorotic streak virus (PCLSVFLt36) promoter with a duplicated enhancer domain for constitutive expression in plants and the pea rbcS 3' sequence for efficient transcript termination (Maiti and Shepherd, *Biochem. Biophys. Res. Commun.*, **244**:440-444 (1998)). The pKLP36 vector also contains a kanamycin resistance gene driven by the *Agrobacterium tumefaciens* NOS promoter to allow for selection of transgenic tissues and plants.

b. Next, the oxygenase gene construct was moved into *Agrobacterium tumefaciens*, strain C58C1, by a modified triparental mating procedure routinely used by the University of Nebraska Plant Transformation Core Research Facility. This involved incubating *E. coli* cells carrying the oxygenase construct with a mixture of the *A. tumefaciens* cells and *E. coli* cells carrying the helper plasmid pRK2013 for plasmid mobilization. *A. tumefaciens* cells containing the oxygenase construct and the helper plasmid were then used to transform tobacco and *Arabidopsis* with the assistance of the University of Nebraska Plant Transformation Core Research Facility. For tobacco, leaf explants were incubated with a suspension of the *A. tumefaciens* cells, and shoots were regenerated on solid medium containing kanamycin (Horsch et al., *Science*, **227**:1229-1231 (1985)). Ten shoots were selected from the transformation experiments, placed on rooting medium for a few weeks, and then moved to pots in the greenhouse. Further details of the transformation of tobacco are provided in attached Exhibit 2, the entire contents of which are incorporated herein by reference. For *Arabidopsis*, a pot of plants with flowers was incubated with a suspension of the *A. tumefaciens* cells, and the plants were then allowed to set seed (Bechtold et al., *C.R. Acad. Sci. Paris, Sciences de la vie/Life Sciences*, **316**:1194-1199 (1993); Clough and Bent, *Plant Journal*, **16(6)**:735-743 (1998)). The seeds were collected and germinated on medium with kanamycin . After the seedlings had developed an adequate root system, ten plants were selected from each transformation experiment and moved to pots in a growth chamber.

c. The expression of the oxygenase gene in the transformed plants was evaluated. Western blots of leaf lysates from several transformed plants were prepared and probed with polyclonal antibodies that detect the oxygenase component of dicamba O-demethylase. The approximately 40 kD oxygenase protein was detected in 100% of the eight tobacco transformants

tested. The results are shown in attached Figure C. In Figure C, each lane contains approximately 40 μg of protein. Lane 1 contains purified oxygenase protein expressed in *E. coli* 3MP, and the remaining lanes contain protein from the tobacco leaf lysates. Also, the expressed oxygenase protein was active in plant cells. Oxygenase activity was assayed by combining leaf lysates from the transformed plants expressing the oxygenase protein with excess amounts of the other O-demethylase components (reductase and ferredoxin) purified from *P. maltophilia*, strain DI-6, as described in Example 1 of the above-referenced application. These mixtures were shown to possess dicamba O-demethylase activity with both a standard ^{14}C-labeled dicamba assay and a HPLC assay that detected 3,6-dichlorosalicylic acid as the product of the dicamba O-demethylase enzymatic activity.

5. I hereby declare that all statements made herein of my own knowledge are true, and that all statements made on information and belief are believed to be true, and further that the statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Date: _____, 2001 By: _____
Dr. Donald P. Weeks